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14. ABSTRACT The project aims to improve our prediction of tumor response for patients with metastatic colorectal cancer (mCRC). Over the past year, we have been refining our tissue slice culture platform through standardization of protocol and modifications to culture conditions. We have developed a "sensitivity" index that allows for meaningful comparison between samples. Detailed analyses of tumor response to drugs are currently underway, along with advanced proteomic studies. Investigations using the slice culture to examine tumor response to immunotherapies have provided valuable insights towards novel strategies in the treatment of mCRC.					
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Annual Report 2017: Tumor Slice Culture: A new avatar for personalized oncology

1. **INTRODUCTION:** The goal of this research is to advance our ability to precisely tailor therapy for solid tumors based on an *ex-vivo* tumor slice culture (TSC) platform as a novel avatar of personalized oncology. The objective of this proposal is to establish a robust, efficient, reproducible platform to interrogate the response of a given tumor to drugs (cytotoxics, kinase inhibitors, immune modulators) and adoptive cellular immunotherapy using human samples.

2. **KEYWORDS:** organotypic, colorectal, metastases, in vitro, drug sensitivity, slice culture, tumor infiltrating lymphocytes (TIL), CAR-T (chimeric antigen receptor T cells), PD-1, kinobeads, RNAseq, kinases.

3. **ACCOMPLISHMENTS:**

Project 1: Tumor Slice Cultures as Predictor of Chemosensitivity

The work in Project 1 was conducted at the University of Washington and Institute for Systems Biology involving the P.I., Raymond Yeung, and co-PI, Qiang Tian.

Specific Aim 1: Testing chemosensitivity using TSC

Major Task 1: Patient selection and enrollment

Over the 12 months, we enrolled 26 patients from the UWMC Liver Tumor Clinic with a diagnosis of colorectal carcinoma with liver metastases who underwent surgical resection. All patients were consented under our IRB approved studies (#31281 and #51710). This rate of enrollment is in keeping with our projected timeline of the proposal.

Major Task 2: Tumor slice cultures

For all 26 cases of resected CRC, we were successful in procuring and establishing slice cultures from the tumors using the method described in our proposal (e.g., 100% success). Our current protocol places the tissue slices in 24-well transwell plates using a defined media under normoxic condition. Baseline viability and growth were determined using the MTS assay without killing the tissues. Baseline MTS values varied from tumor to tumor as expected due to tumor heterogeneity and differences in pre-operative treatments. Indeed, the viability scores significantly correlated with pathologic assessment of tumor viability/necrosis.

We continue to optimize the conditions for TSC by manipulating growth factors and nutrients. Compared to other histologies, TSC derived from CRC are relatively robust in terms of their growth in vitro. As such, we interrogated the slices using various drugs (see below), and developed a 'sensitivity' index based on both viability and toxicity of the slices following exposure to treatments. We are in the process of assessing the pathologic outcome using H&E staining of the treated slices and correlating the results with our index.

To date, we have had 100% success in creating organotypic cultures from CRC liver metastases. We have not begun our microfluidic experiments since we are still modifying and optimizing conditions.

Major Task 3: Drug treatments and responses

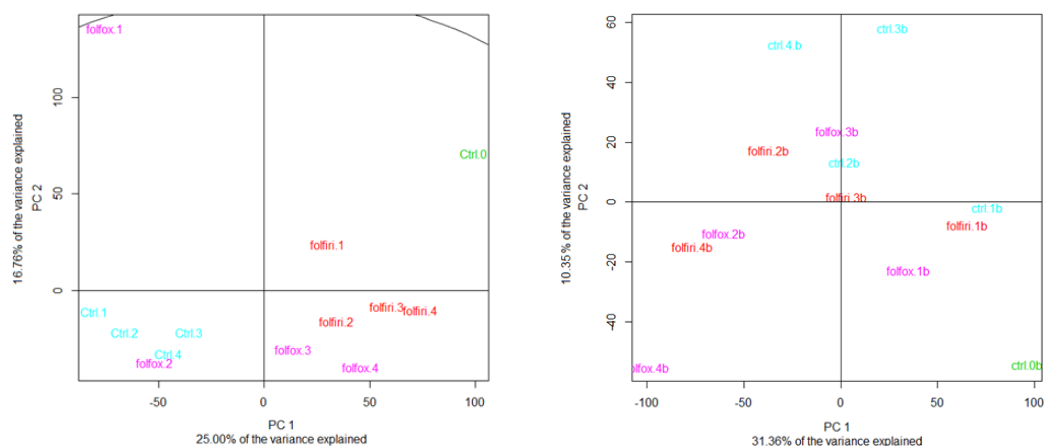
Significant efforts have been devoted to standardizing the protocol for drug sensitivity testing including timing (e.g., when to start, treatment duration, re-dosing regimen based on drug half-lives). We have adopted a shorter treatment period (e.g., 3-4 days) to optimize the ‘signal’ related to drug effects vs. deterioration of the slices in vitro. During this reporting period, we have tested the TSCs for their responses to two standard-of-care chemotherapy regimens for CRC: FOLFOX and FOLFIRI. There is currently no clear clinical guidance for choosing which regimen for CRC patients. Identifying molecular signatures distinguishing the two treatments will have important clinical implication for guiding drug selection. The TSCs were treated with 5FU (1µg/ml) alone or in combination with Oxaliplatin (1µg/ml, FOLFOX) or Irinotecan (2µg/ml, FOLFIRI) for 24, 48, 72 and 96 hours, alongside with a DMSO-treated control at each time point as well as a no-treatment day 0 control. Following drug treatments, TSCs were transferred to RNAlater stabilizing solution for downstream RNA/DNA extraction and molecular analyses.

Specific Aim 2: Correlation with clinical and molecular data

Major Task 1: Collect clinical and molecular data

We collect clinical data including patients’ demographics, details of metastatic disease, prior treatments and responses, tumor markers (CEA), pathologic findings (treatment effects, tumor grade), as well as molecular data including mutation analyses of RAS, BRAF and MMR status for all patients participating in this study.

Separately, we are generating new molecular data from TSCs undergoing treatment in vitro. Specifically, we (led by Dr. Qiang Tian) performed RNAseq on tumor slices following various treatments to examine the molecular ‘response’ of the drugs. To do so, we optimized the protocol and QC to generate an average of 10-20 million reads from each of the TSC’s. Based on a very small sample size, we are able to separate different CRCs based on their changes in RNA expression patterns following drug exposure (see Figure). Further validation is currently underway.



PCA analysis of RNA-seq data generated from TSCs of 2 different CRCs.

Major Task 2: Statistical analyses

This is deferred until the sample size is sufficiently large to conduct meaningful analyses.

Project 2: Kinase inhibition in metastatic colorectal carcinoma

This Project is led by Qiang Tian at ISB, working in collaboration with Shao-En Ong and Raymond Yeung at UW.

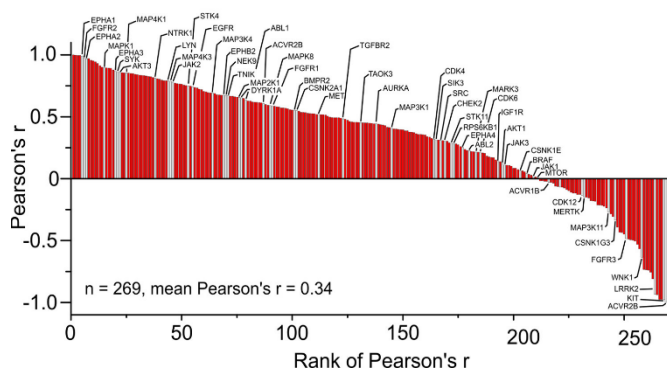
Specific Aim 1: Kinase identification in mCRC

Major Task 1: kinobead assay

Several groups have applied kinobeads to profile kinase inhibitors and in studies of cell signaling, but they have not been routinely applied in analyses of clinical samples. Most published kinobead protocols require > 25 mg of protein, precluding routine application to clinical diagnostics. Our technology takes advantage of the kinobeads' enrichment of the kinome to increase analytical coverage of these important and often low abundance enzymes. By combining kinase enrichment with phospho-profiling, we can quantify protein abundances for 368 of 518 human kinases and quantify many known, as well as novel, phosphorylation sites on these important regulatory enzymes; we use annotations of known regulatory phosphosites from PhosphoSitePlus (CST) and pathway information from the Reactome database to map our data to active signaling pathways. Our current protocol uses $\leq 300 \mu\text{g}$ of protein for kinase enrichment and 1 mg of protein for the phosphopeptide enrichment workflow, with LC-MS analyses on an Orbitrap Elite MS instrument.

We (led by Shao-En Ong) have been validating our kinobead platform's ability to identify druggable kinase-dependent signaling pathways. We applied our kinobeads to a panel of 11 CCLE cancer cell lines and identified a total of 1164 proteins with 4870 phosphosites, including 2300 phosphosites on 299 protein and lipid kinases. Comparing kinase mRNA and protein abundances in this panel, we found that the correlation of relative differences of mRNA and protein expression levels in our dataset varied considerably between individual kinases, ranging from very strong positive correlation (TNK1, $r = 0.95$) to very strong negative correlation (KIT, $r = -0.99$). (see Figure) Our correlation analysis includes 54 of the 91 recently proposed "cancer driver kinases", highlighting the relevance of our kinobead approach in the interpretation of kinome expression data from human cancer cell lines/tumor samples.

Moving forward, we propose to use an Orbitrap Fusion Lumos instrument, which will increase the sensitivity by $\sim 20\times$ and $> 2\times$ number of peptides identified per analysis due to the brighter luminosity on the source and faster scan speeds on the newer instrument. We have just acquired this instrument through a separate funding mechanism.



which covers ~250 cancer-related genes. We have deferred these analyses on clinical samples until we have optimized our proteomic platform using our new instrument.

Specific Aim 2: Test kinase inhibitors using TSC

Major Task 1: Kinase inhibition in TSC

Not yet begun.

Major Task 2: Evaluate hepatotoxicity of inhibitors

We have assessed viability of non-tumor adjacent liver (NTL) slices obtained from resected specimens demonstrating feasibility of testing human liver slices for drug-induced toxicities. Similar to the tumor slices, NTL cultures remain viable for >1 week based on MTS assay. These slices undergo an acute phase response, which recovers in ~4 days based on changes in expression profile of acute phase proteins. With this understanding, we will be better prepared to interpret the results of hepatotoxicity following exposure to kinase inhibitors or other drugs.

Project 3: Immune evasion in mCRC

This project is under the direction of Venu Pillarisetty as PI, and Nick Crispe as co-PI, both at the University of Washington.

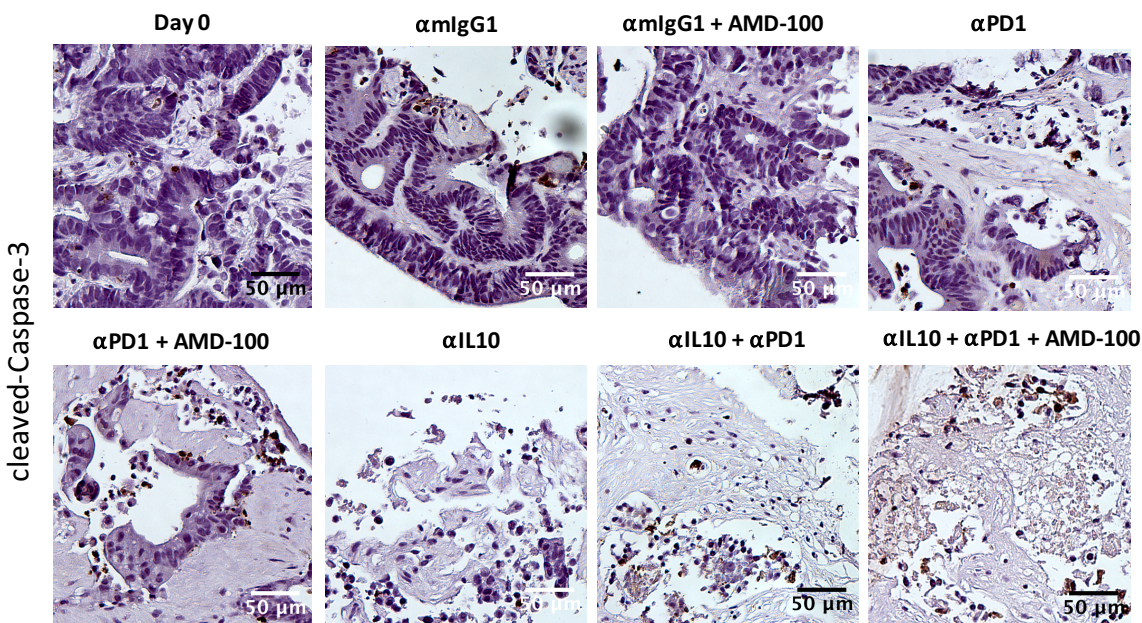
Specific Aim 1: Optimizing TIL function

Major Task 1: Define immunosuppressive molecules

The major activities and specific objectives that we addressed during the first 12-month period of the project centered around optimization of the slice culture model system and testing of well-described immune checkpoint inhibitors on TIL function. All of the Major Tasks and Subtasks in Project 3 were planned to run concurrently throughout the first 30 months of the project. We have spent the past year optimizing a 6-color multiplexed immunohistochemistry panel, which we are now ready to use to optimally detect immunosuppressive signals within the colorectal cancer liver metastasis tumor microenvironment. We have also worked to obtain the CAR-T cells to be used in the slice culture system, and close to being able to transfer those reagents to the University of Washington.

Major Task 2: Effects of checkpoint inhibition on TILs

We have created TSC from a total of 12 CRCLM tumors during this project period and have been successful in evaluating the response of TIL to immune checkpoint inhibitor therapy. We have already noted striking results from these studies, with regards to combinations of therapies that block immunosuppressive signals (see Figure). Notably, we have found that combination of PD-1 blockade and CXCR4 blockade accelerates carcinoma cell death in TSC. We have also found that blockade of IL-10 similarly destroys the tumor in culture. Our preliminary evaluation of cytokine levels in the culture supernatant confirm that the cytotoxic effect appears to be T-cell mediated. These findings are the results of several replicate experiments and we are working to fully define the mechanism of action.



CRCLM TSC cultured with combination immunotherapies demonstrate the ability to activate immunosuppressed TIL.

Specific Aim 2: Optimizing CAR-T cell function

These studies have not begun.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interests?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We have made significant progress over this reporting period, and we will continue with the planned experiments as outlined in the original proposal and SOW.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Shortly after the study began, one investigator, Dr. Edward Lin, left the Institution. His primary role was to help recruit patients onto the study through his clinical trial. This effort has been replaced by our increased clinical volume through the Secondary Liver Tumor Clinic at the University of Washington Medical Center. Consequently, there has been very little impact of his departure towards enrollment of patients to the current study. Indeed, based on our projected enrollment, we are ahead of target at the Year 1 mark. We do not anticipate any enrollment issues for the remainder of the study.

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

N/A

6. PRODUCTS:

Publications, conference papers, and presentations

Nothing to report

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on this project?

No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

There are 3 projects that make up this Award, each with its own P.I., working closely together to maximize scientific engagement and productivity. The overall P.I., Raymond Yeung (University of Washington), is largely responsible for the work performed in Project 1 with the assistance of a Co-P.I., Qiang Tian. Project 2 is headed by Qiang Tian at the Institute for Systems Biology working closely with Shao-En Ong (University of Washington). Venu Pillarisetty (University of Washington) heads the third Project. Each of the P.I. and Co-P.I. crafted the progress reports for their respective Projects and are glued together as a single document.

9. APPENDICES

none